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RESEARCH PAPER

Differential signalling in human cannabinoid CB_1 receptors and their splice variants in autaptic hippocampal neurones

Alex Straiker, Jim Wager-Miller, Jacqueline Hutchens and Ken Mackie

Department of Psychological and Brain Sciences, Gill Center for Biomolecular Science, Indiana University, Bloomington, IN, USA

Correspondence
Alex Straiker, Department of Psychological and Brain Sciences, Gill Center for Biomolecular Science, Indiana University, Bloomington, IN 47405, USA.
E-mail: straiker@indiana.edu

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BACKGROUND AND PURPOSE

Cannabinoids such as Δ^9- tetrahydrocannabinol, the major psychoactive component of marijuana and hashish, primarily act via cannabinoid CB_1 and CB_2 receptors to produce characteristic behavioural effects in humans. Due to the tractability of rodent models for electrophysiological and behavioural studies, most of the studies of cannabinoid receptor action have used rodent cannabinoid receptors. While CB_1 receptors are relatively well-conserved among mammals, human CB_1 (hCB_1) differs from rCB_1 and mCB_1 receptors at 13 residues, which may result in differential signalling. In addition, two hCB_1 splice variants (hCB_{1a} and hCB_{1b}) have been reported, diverging in their amino-termini relative to hCB_1 receptors. In this study, we have examined hCB_1 signalling in neurones.

EXPERIMENTAL APPROACH

hCB_1, hCB_{1a} hCB_{1b} or rCB_1 receptors were expressed in autaptic cultured hippocampal neurones from $CB_1^{-/-}$ mice. Such cells express a complete endogenous cannabinoid signalling system. Electrophysiological techniques were used to assess CB_1 receptor-mediated signalling.

KEY RESULTS

Expressed in autaptic hippocampal neurones cultured from $CB_1^{-/-}$ mice, hCB_1, hCB_{1a} and hCB_{1b} signal differentially from one another and from rodent CB_1 receptors. Specifically, hCB_1 receptors inhibit synaptic transmission less effectively than rCB_1 receptors.

CONCLUSIONS AND IMPLICATIONS

Our results suggest that cannabinoid receptor signalling in humans is quantitatively very different from that in rodents. As the problems of marijuana and hashish abuse occur in humans, our results highlight the importance of studying hCB_1 receptors. They also suggest further study of the distribution and function of hCB_1 receptor splice variants, given their differential signalling and potential impact on human health.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

AEA, arachidonoyl ethanolamide; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; THC, tetrahydrocannabinol; 2-AG, 2-arachidonoyl glycerol

Introduction

The cannabinoid CB_1 receptor is the chief mediator of the CNS effects of cannabinoids (Howlett *et al.*, 2002; receptor nomenclature follows Alexander *et al.*, 2011). It is these receptors that are engaged by phytocannabinoids such as Δ^9- tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of marijuana and hashish (Gaoni and

Mechoulam, 1964). An understanding of the function of these receptors is critical to understanding the nature of this psychoactivity as well as potential therapeutic consequences of CB_1 receptor activation. In addition to CB_1 receptors, the endogenous cannabinoid signalling system consists of an assortment of proteins that have been proposed to play roles in the production, transport and breakdown of endogenous cannabinoids (endocannabinoids). Taken together, these proteins form a constellation of cannabinoid-related signalling proteins and potential sites of study and therapeutic manipulation (Kano *et al.*, 2009). Much of this machinery is expressed in cultured autaptic hippocampal neurones, which make them an attractive model system to study the molecular details of endocannabinoid signalling. These neurones have both presynaptic CB_1 receptors that modulate neurotransmitter release, and the enzymes involved in endocannabinoid production and degradation. In particular, they express the enzymes involved in the synthesis and degradation of 2-arachidonoyl glycerol (2-AG) (Stella *et al.*, 1997), which is synthesized in response to depolarization or activation of selected G_q-coupled receptors (Straiker and Mackie, 2005; 2007). Because transfection of CB_1 receptors into neurones cultured from mice genetically lacking CB_1 receptors ($CB_1^{-/-}$ mice) rescues endogenous retrograde cannabinoid signalling, these cultures present a unique opportunity to investigate the function of CB_1 receptors.

For many good reasons, including the genetic pliability of the mouse, most studies examining the neuronal effects of CB_1 receptor signalling have made use of rodent models. CB_1 receptors are well conserved among mammals; the human receptor differs by only a few percent (13 residues out of 473) from mouse and rat CB_1 receptors (the latter two differ from one another by only a single residue) (Matsuda *et al.*, 1990; Gerard *et al.*, 1991). Still, because the societally relevant psychoactivity of exogenous cannabinoids occurs via human, not rodent, CB_1 receptors, it is essential to ascertain whether the signalling properties of hCB$_1$ receptors differ from those of the better-studied mouse and rat CB_1 receptors. As has been shown for many GPCRs, including CB_1 receptors, substitution of even a single residue may substantially alter the signalling properties of a receptor (Song *et al.*, 1999). hCB$_1$ receptors differ from rodent CB_1 receptors at 13 residues, chiefly in the extracellular portions but also at two sites in the carboxy-terminus. Previous studies of heterologously expressed hCB$_1$ receptors have demonstrated that they are functional and their signalling properties are grossly similar to rodent CB_1 receptors (Gerard *et al.*, 1991; Felder *et al.*, 1992; 1993; 1995; Song and Bonner, 1996; Bouaboula *et al.*, 1997; Landsman *et al.*, 1997; 1998; Pan *et al.*, 1998; Guo and Ikeda, 2004; Won *et al.*, 2009). However, we are not aware of any studies that have compared the ability of hCB$_1$ receptors to inhibit synaptic transmission relative to rodent CB_1 receptors. Further complicating the picture, two splice variants of hCB$_1$ receptors have been identified, hCB$_{1a}$ (Shire *et al.*, 1995; Rinaldi-Carmona *et al.*, 1996) and hCB$_{1b}$ (Ryberg *et al.*, 2005; Xiao *et al.*, 2008). Both hCB$_{1a}$ and hCB$_{1b}$ mRNAs are expressed in assorted tissues, including brain, albeit at low levels. While these splice variants were found to share some qualities with hCB$_1$ receptors, they also exhibited unusual properties. Ryberg *et al.* (2005) found that of four candidate endocannabinoids tested (arachidonoyl ethanolamide, 2-AG, noladin ether and virhodamine) only 2-AG bound and activated hCB$_{1a}$ or hCB$_{1b}$ receptors. More surprisingly, 2-AG acted as an inverse agonist, though a more recent study examining hCB$_1$ splice variants expressed in CHO cells failed to confirm this finding (Xiao *et al.*, 2008). Thus, the signalling properties of 2-AG at the hCB$_1$ receptors splice variants, particularly in neurones, remains an unresolved question of considerable interest.

Expression of hCB$_1$ receptors and the splice variants in autaptic hippocampal neurones offers a unique opportunity to observe their functional role in endogenous cannabinoid signalling under otherwise identical conditions. Taking this approach, we have found that the hCB$_1$ receptor splice variants exhibit signalling properties, as measured by electrophysiological methods, that are distinct from one another as well as from rodent CB_1 receptors.

Methods

Culture preparation

All animal care and experimental procedures used in this study were approved by the Animal Care Committee of the Indiana University and conformed to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse hippocampal neurones isolated from the CA1–CA3 region were cultured on microislands as described previously (Furshpan *et al.*, 1976; Bekkers and Stevens, 1991). Neurones were obtained from animals (age postnatal day 0–2) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high-glucose (20 mM) medium containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than 3 h after removal from culture medium.

Electrophysiology

When a single neurone is grown on a small island of permissive substrate, it forms synapses – or 'autapses' – onto itself. All experiments were performed on isolated autaptic neurones. Whole-cell voltage-clamp recordings from autaptic neurones were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 30 glucose and 20 HEPES. Continuous flow of solution through the bath chamber (~2 mL·min^{-1}) ensured rapid drug application and clearance. Drugs were typically prepared as stocks then diluted into extracellular solution at their final concentration and used on the same day.

Recording pipettes of 1.8–3 MΩ were filled with (in mM) 121.5 K gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 MgATP and 0.5 LiGTP. Access resistance and holding current were monitored, and only cells with both stable access resistance and holding current were included for data analysis. A conventional stimulus protocol was followed: the membrane potential was held at –70 mV, and excitatory postsynaptic currents (EPSCs) were evoked every 20 s by triggering an unclamped action current with a 1.0 ms depolarizing step. The resultant evoked waveform

consisted of a brief stimulus artifact and a large downward spike representing inward sodium currents, followed by the slower EPSC. The size of the recorded EPSCs was calculated by integrating the evoked current to yield a charge value (in pC). Calculating the charge value in this manner yields an indirect measure of the amount of neurotransmitter released while minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (the soma). Data were acquired at a sampling rate of 5 kHz.

Induction of depolarization induced suppression of excitation (DSE): after establishing a 10–20 s 0.5 Hz baseline, DSE was evoked by depolarizing to 0 mV for 1–10 s, followed by resumption of a 0.5 Hz stimulus protocol for 10–80+ s until EPSCs recovered to baseline values.

2-AG, the probable endogenous mediator of DSE in these cultures, was applied at 5 μM since this concentration was found to correspond to maximal DSE in autaptic cultures (Straiker and Mackie, 2005).

Neuronal transfection

We transfected neurones using a calcium phosphate-based method adapted from Jiang *et al*. (2004). Briefly, plasmids for the protein of interest and enhanced yellow fluorescent protein (EYFP) or mCherry (2 μg per well) were combined with 2 M $CaCl_2$ in water and gradually added to HEPES-buffered saline (HBS); the mixture was added to the serum-free neuronal media. Coverslips were incubated with this mixture for 2.5 h, while extra serum-free media was placed in a 10% CO_2 incubator to induce acidification. At the end of 2.5 h, the reaction mixture was replaced with acidified serum-free media for 20 min. After this, cells were returned to their home wells. Each data set was taken from at least three different neuronal platings.

Western blot

HEK293 cells were grown to approximately 90% confluency in six-well dishes. rCB_1 or hCB_1, CB_{1a} or CB_{1b} receptor expression plasmids were transfected into these cells using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Transfected cells were grown overnight. The next day, they were removed from the incubator and chilled on ice for 5 min. Following a wash with ice-cold 1X PBS, cells were covered with 200 μL lysis buffer [100 mM Tris (pH 7.4), 150 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 6 mM $MgCl_2$ and 100 mM PMSF] and incubated on ice 5 min. Cells were then scraped, and lysates were sonicated and spun down at 10 000× *g* and 4°C. The supernatant was collected, and protein concentration was determined using the Bradford assay. The samples were normalized to total protein, and 25 μg protein of each sample was run on a 4–12% Nu-Page gel. The separated proteins were transferred to nitrocellulose, and Western blots were performed using a rabbit polyclonal anti-hCB_1 receptor antibody (raised against the first 100 amino acids of hCB_1) and a mouse monoclonal anti-HA11 (Cat# MMS-101P, CRP Inc., Berkeley, CA, USA). Primary antibodies were diluted 1:1000 in Odyssey blocking buffer (Li-cor Biosciences, Lincoln, NE, USA). Secondary antibodies used included a donkey anti-rabbit conjugated with an IR800 dye (Cat# 605-732-002, Rockland Inc., Gilbertsville, PA, USA) and a goat anti-mouse conjugated with an IR680 dye (Cat# A21057, Invitrogen). Both were diluted 1:5000 in a 50:50 mixture of 1× PBS and Odyssey blocking buffer. Western blots were scanned on an Odyssey near IR scanner, and images were processed using Photoshop CE.

Lanes were drawn and plots were made using ImageJ from NCBI. Background was subtracted from plots, and the area under the curve was determined for each CB1-expressing sample.

Densitometry

HEK293 cells were transfected using Lipofectamine 2000 as per manufacturer's protocol (Invitrogen). After a 24 h incubation period, cells were transferred onto poly-D lysine-coated coverslips and allowed to attach overnight. Cultures were fixed, incubated in blocking buffer (1× PBS, 5% donor donkey serum, 0.1% saponin) and then treated with mouse anti-HA11 antibody (Covance Research Products, Inc., Berkeley, CA, USA) at a 1:1000 dilution. The secondary antibody used was FITC-conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) used at 1:150 dilution. Images were collected on a Nikon Eclipse TE2000-E (Melville, NY, USA) using Metamorph software. To calculate membrane –associated and total CB_1 receptor immunoreactivity, a rectangular region of interest (ROI) was drawn perpendicular to the plasma membrane using ImageJ software from NCBI. ROIs included the cytosol and the area outside cells. Intensity plots were generated, and background (intensity in the area outside of the cell) was subtracted. The intensity corresponding to the region of the plasma membrane was divided by the total intensity in the ROI to determine the percent of CB_1 receptors on the membrane. Data were collated on Excel (Microsoft, Redmond, WA, USA) and analysed using Prism 4 software (GraphPad Software, San Diego, CA, USA).

Immunocytochemistry

Cultured neurones were fixed in 4% paraformaldehyde for 30–60 min, washed, treated with a detergent (Triton-X100, 0.3% or saponin, 0.1%) and milk (5%) in PBS, followed by mouse anti-HA11 antibody overnight at 4°C. Secondary antibodies (Alexa 488, 1:500, Invitrogen, Inc.) were subsequently applied at room temperature for 1.5 h. Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using Leica LAS AF software and a 63× oil objective. Images were processed using ImageJ (available at http://rsbweb.nih.gov/ij/) and/or Photoshop (Adobe Inc., San Jose, CA, USA). Images were modified only in terms of brightness and contrast.

Materials

$CB_1^{+/-}$ mice to found a $CB_1^{-/-}$ colony were generously provided by Catherine Ledent *et al*. (1999). The rCB_1 and hCB_1 plasmids have been previously described (Mackie *et al*., 1995; Xiao *et al*., 2008). hCB_1 plasmids were the generous gift of Tung Fong (Merck, Whitehouse Station, NJ, USA). WIN 55212-2 (WIN) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-AG, and arachidonoyl ethanolamide (AEA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). SR 141716 was obtained from NIDA Drug-Supply Program (Bethesda, MD, USA).

Results

Expression and characterization of rCB$_1$, hCB$_1$ and hCB$_1$ receptor splice variants

In order to examine the functionality of human CB$_1$ receptors and the splice variants, relative to one another as well as relative to the more commonly studied rat CB$_1$ receptors, we first examined transient expression of these constructs in the HEK293 cell line by Western blotting.

In principle, any observed difference in response profiles for hCB$_{1a}$ and hCB$_{1b}$ versus hCB$_1$ receptors and for hCB$_1$ versus rCB$_1$ receptors might be due to different levels of expression. For example, if hCB$_1$ receptors were expressed to a lesser extent than rCB$_1$ receptors or the shorter splice variants, and signalling was proportional to receptor number (i.e. no 'spare' receptors), one might observe a diminished response profile. Using densitometry, we found that rCB$_1$, hCB$_1$ and hCB$_{1a}$ receptors were all expressed at similar levels. However, hCB$_{1b}$ receptors were expressed at 2.5-fold higher levels than hCB$_1$ receptors (Figure 1A,B; $P < 0.01$, one-way ANOVA with Dunnett's *post hoc* test).

Inhibition of synaptic transmission by CB$_1$ receptors is likely to require that these receptors are appropriately trafficked to the cell surface. Thus, if hCB$_1$ receptors were more poorly trafficked to the membrane, this could account for any observed differences in signalling. To investigate this, we examined membrane-localized receptor labelling as a percentage of total labelling. We found that all four CB$_1$ receptors expressed to a similar degree at the membrane (measured as the ratio of membrane CB$_1$ immunoreactivity to total immunoreactivity in HEK293 cells; Figure 1C,D). We also confirmed immunocytochemically that the three receptors were expressed and trafficked normally in transfected neurones (Figure 2). We found that hCB$_1$, hCB$_{1a}$ and hCB$_{1b}$ receptors were all robustly expressed in neurones. Therefore, impaired receptor expression or trafficking is unlikely to account for any differences in signalling in the autaptic cultures between hCB$_1$ and rCB$_1$ receptors.

Transfection of rCB$_1$ receptors into CB$_1^{-/-}$ neurones fully rescues DSE responses

A simple way to quantify DSE and thereby assess CB$_1$ receptor signalling is to assemble a 'depolarization–response curve' showing EPSC inhibition in response to increasing durations of depolarization (Straiker and Mackie, 2005; 2009). Cells are depolarized for increasing durations (50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s), resulting in increasing synthesis of endocannabinoids (probably 2-AG; Straiker and Mackie, 2005; Figure 3). The resulting inhibition can be measured and analysed in a manner very similar to a classical dose–response curve. Using this method, we find that transfection of rCB$_1$ receptors into CB$_1^{-/-}$ neurones fully rescued the DSE responses (Figure 3C).

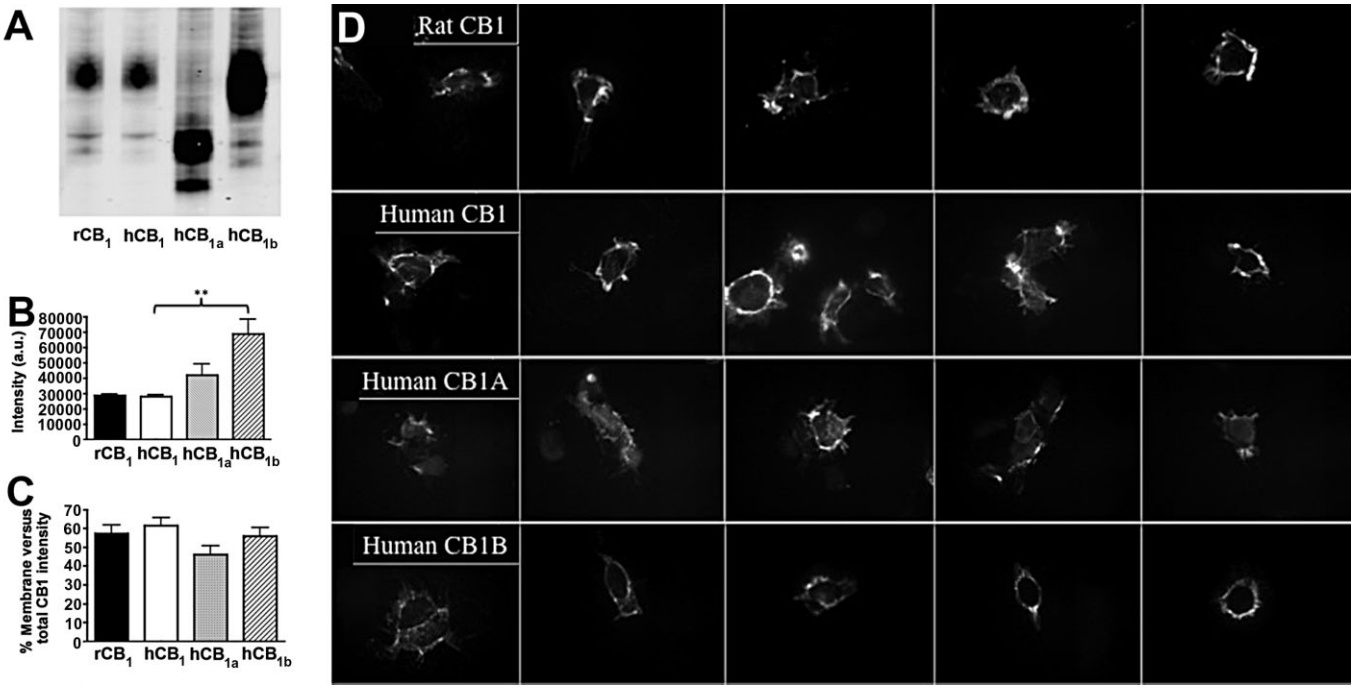

Figure 1

Expression of hCB$_1$receptors, the splice variants and rCB$_1$ receptors in HEK293 cells. (A) Representative Western blot shows bands for HA staining of rCB$_1$, hCB$_1$, and splice variants hCB$_{1a}$ and hCB$_{1b}$, transiently expressed in HEK293 cells. (B) Densitometry measurement of Western blots typical of those shown in panel A indicates that hCB$_{1b}$ is expressed at higher levels than hCB$_1$. *X*-axis arbitrary units (a.u.) ($n = 4$ independent experiments). (C) Membrane expression as a percentage of total CB$_1$ immunoreactive intensity in HEK293 cells indicates that there is no significant difference in surface expression between the different receptors ($n = 20$). (D) Sample images of HEK293 cells transfected with rCB$_1$, hCB$_1$, and splice variants hCB$_{1a}$ and hCB$_{1b}$. Scale bar = 10 μm $^{**}P < 0.01$ one-way ANOVA with Dunnett's *post hoc* test versus hCB$_1$.

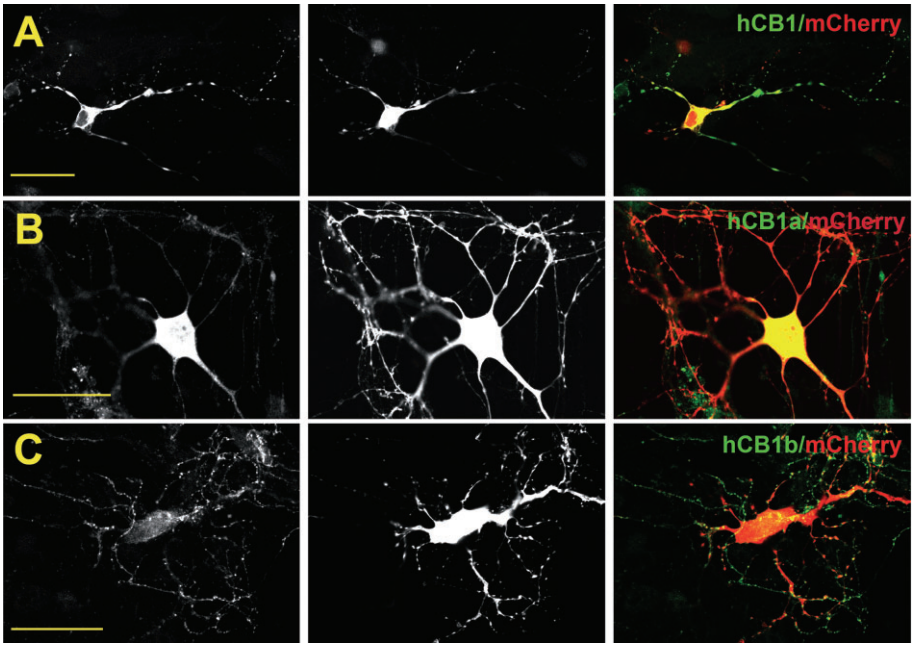

Figure 2

Expression of hCB_1 receptors and the splice variants in neurones. (A) Left panel shows HA staining for hCB_1 expression in an autaptic hippocampal neurone transfected with HA-hCB_1. Centre panel shows mCherry for the same neuron. Right panel shows overlay (hCB_1 = green, mCherry = red, overlap = yellow). (B) Staining as in panel A for hCB_{1a}. (C) Staining as in panel A for hCB_{1b}. Scale bars = 25 μm.

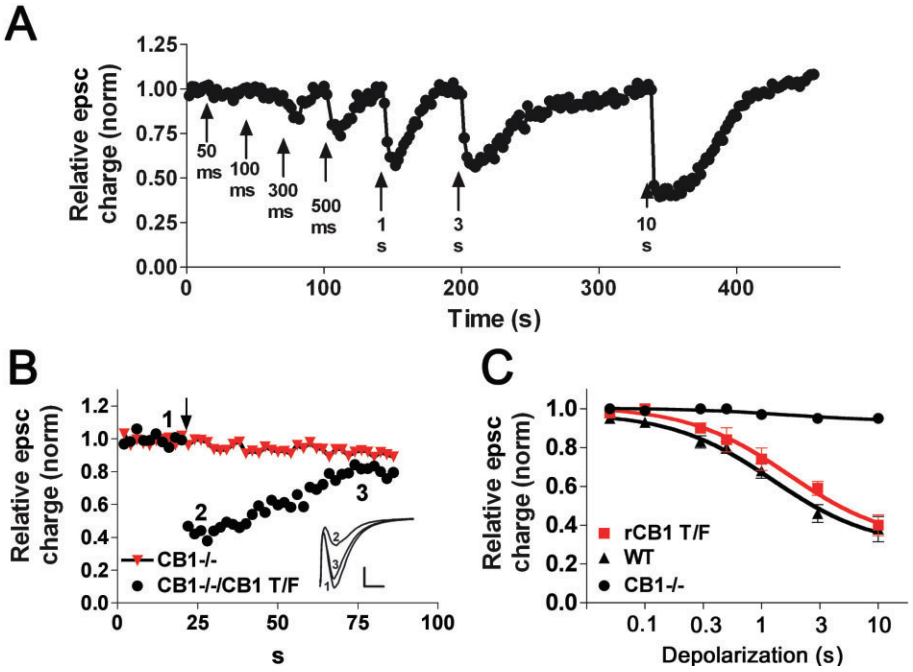

Figure 3

Transfection of rCB_1 receptors into $CB_1^{-/-}$ neurones fully rescues DSE. (A) Sample time course in wild-type cultured mouse hippocampal neurones showing EPSC charge in response to a series of depolarizations of increasing duration (50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s). (B) Typical DSE time course of a rCB_1-transfected neurone (CB1 T/F) and a non-transfected $CB_1^{-/-}$ neurone in response to a 3 s depolarization; inset shows sample EPSCs at the time points indicated for transfected neurone (1, control, 2, peak DSE, 3, recovery). (C) DSE depolarization–response curves, representing progressive inhibition in response to increasing durations of depolarization in wild type (WT), $CB_1^{-/-}$ cells and $CB_1^{-/-}$ cells transfected with rCB_1 receptors. Data for $CB_1^{-/-}$ (from Straiker and Mackie, 2005) is included for reference.

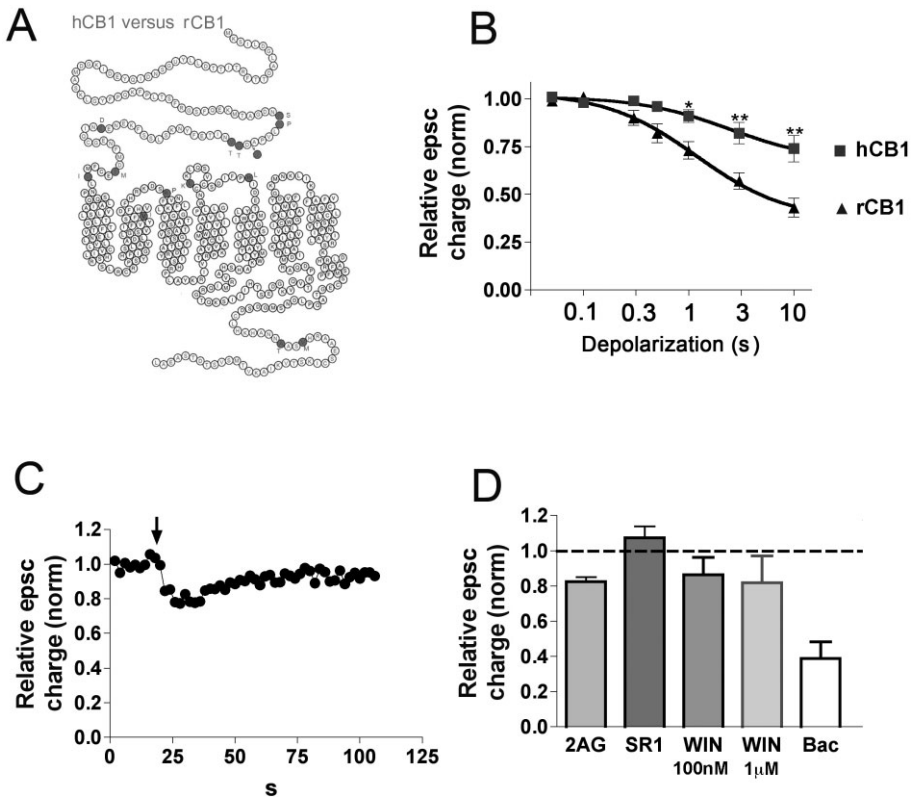

Figure 4

hCB$_1$ receptors signal less robustly than rCB$_1$ receptors. (A) Helixnet diagram shows the structure of the hCB$_1$ receptor, with residues different from rCB$_1$ receptors shown in darker symbols. (B) DSE depolarization–response curve, representing inhibition in response to increasing durations of depolarization (50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s) in cells transfected with rCB$_1$ receptors or with hCB$_1$ receptors. *P < 0.01; **P < 0.001, two-way ANOVA with Bonferroni *post hoc* test. (C) Typical DSE time course of an hCB$_1$ receptor-transfected neurone in response to a 3 s depolarization (arrow). (D) Bar graph shows responses to endocannabinoid 2-AG (5 μM), the CB$_1$ receptor antagonist SR1 (200 nM), the synthetic CB$_1$ agonist WIN (100 nM and 1 μM) and the GABA$_B$ receptor agonist baclofen (Bac; 25 μM) in hCB$_1$ receptor-transfected neurones.

hCB$_1$ receptors signal poorly relative to rCB$_1$ receptors

As shown in a figure adapted from Bramblett (Figure 4A) (Bramblett *et al.*, 1995), the structure of hCB$_1$ receptors differs from that of rCB$_1$ receptors at only 13 residues. To investigate hCB$_1$ receptor signalling, these receptors were transfected into autaptic hippocampal neurones cultured from CB$_1^{-/-}$ mice.

Notably, we found that hCB$_1$ receptors signalled very poorly relative to rCB$_1$ receptors Figure 4B shows that even for a 10 s depolarization, only ~20% of the EPSC was inhibited in hCB$_1$-expressing neurones, while ~50% of the glutamate release was inhibited in rCB$_1$-expressing neurones. One explanation for this result is that hCB$_1$ receptors less efficiently stimulate the signalling that suppresses glutamate release. Indeed, this seems to be the case as 5 μM 2-AG only inhibited the EPSC charge by ~20% (Figure 4C) in hCB$_1$-expressing neurones whereas, in rCB$_1$-transfected neurones, the inhibition was greater (relative EPSC charge, 0.51 ± 0.10, n = 5, P < 0.05, unpaired t-test). Similarly, AEA (5 μM) also signalled poorly (relative EPSC charge, 0.93 ± 0.08; n = 4) at a concentration that we have previously found to robustly inhibit EPSCs (Straiker and Mackie, 2005). Another explanation for impaired hCB$_1$ receptor signalling might be that these receptors exhibited a high level of constitutive activity. In that case, activation by exogenous agonists of hCB$_1$ receptors would appear less efficacious as the receptors are already active. To assess whether this is the case, we treated cells with the hCB$_1$ receptor inverse agonist, SR141716 (SR1, 200 nM) (Ryberg *et al.*, 2005). If hCB$_1$ receptors had significant constitutive activity, we would expect to see an enhancement of EPSC size. However, we found that SR1 treatment did not increase EPSC size in hCB$_1$-transfected neurones (Figure 4D), suggesting that the reduced signalling that we observed with transfected hCB$_1$ receptors was not due to excessive constitutive activation. In principle, it is also possible that diminished hCB$_1$ receptor signalling was due to a general interference with G-protein-mediated signalling after over-expression of this receptor. If so, one would expect a similar interference with modulation of neurotransmission by other GPCRs such as the GABA$_B$ receptor (Straiker *et al.*, 2002). However, we found that treatment with the GABA$_B$ agonist baclofen (25 μM) substantially inhibited neurotransmission in hCB$_1$-transfected neurones (Figure 4D), similar to effects in non-transfected autaptic neurones (Straiker *et al.*, 2002), indicating that global GPCR presynaptic inhibition remains intact in neurones transfected with hCB$_1$ receptors.

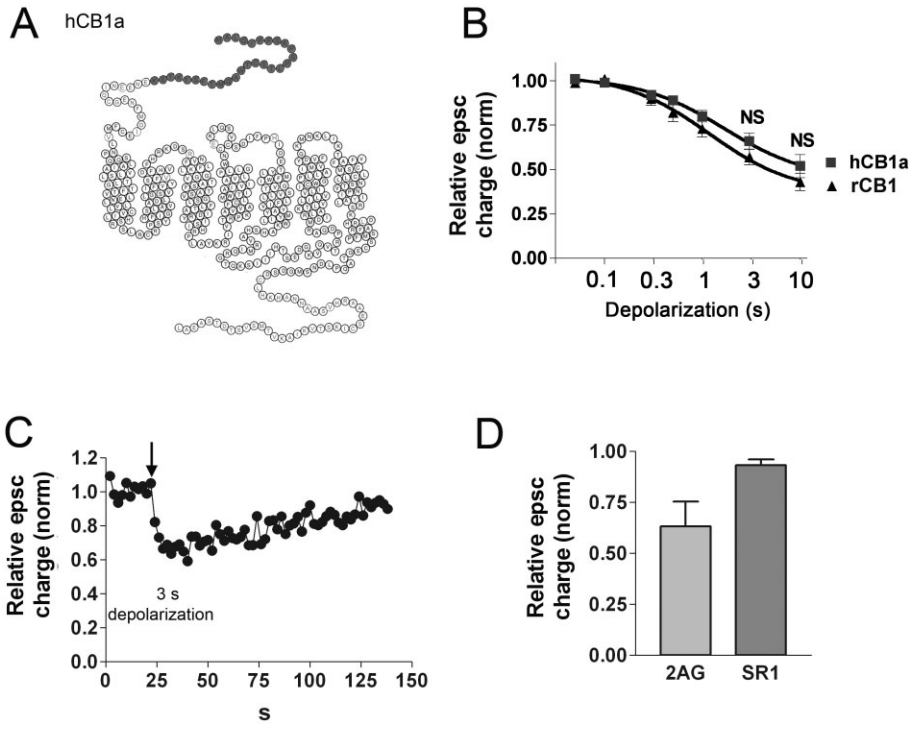

Figure 5

hCB_{1a} receptor signalling is more robust than hCB_1 receptor signalling. (A) Helixnet diagram shows hCB_{1a} with the substituted amino terminus residues added to provide a schematic representation of the differences relative to rCB_1 receptors. (B) DSE depolarization-response curve, representing inhibition in response to increasing durations of depolarization (50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s) in neurones transfected with hCB_{1a} receptors was not different from that in cells transfected with rCB_1 receptors. NS, two-way ANOVA with Bonferroni *post hoc* test. rCB_1 curve is shown for reference. (C) Typical DSE time course in response to 3 s depolarization in a neurone expressing hCB_{1a} receptors. (D) Bar graph shows summary responses to the endocannabinoid 2-AG (5 μM) and CB_1 receptor antagonist SR1 (200 nM) in neurones expressing hCB_{1a} receptors.

It is possible that hCB_1 receptors were activated in a ligand-selective manner whereby, for instance, 2-AG activated the receptor inefficiently in comparison to other ligands. For example, Glass and Northup (1999) showed that Δ^9-THC, HU-210 and WIN differently activated GTPγS binding via G_i- and G_o-proteins, with HU210 activating both G_i and G_o strongly, Δ^9-THC doing so weakly, and WIN activating one strongly and one moderately. To assess ligand specificity at hCB_1, relative to rCB_1 receptors, we tested the synthetic CB_1 receptor agonist WIN at 100 nM and 1 μM. However, we found that WIN activation of hCB_1 receptors was similarly diminished at both concentrations (Figure 4D).

hCB_{1a} receptors signal more robustly than hCB_1 receptors

The structures of the splice variants hCB_{1a} and hCB_{1b} differ substantially from that of hCB_1 receptors. In the case of hCB_{1a}, a frame shift introduces 29 new amino terminal residues and retains only six residues of the original hCB_1 amino terminus, considerably shortening the amino terminus. Interestingly, these changes enhanced hCB_{1a} receptor signalling in autaptic neurones so that it was nearly indistinguishable from that of rCB_1 receptors (Figure 5B,C). Similarly, 5 μM 2-AG produced a substantial inhibition of EPSCs in hCB_{1a} expressing cells (Figure 5D). These findings contrast with the findings of Ryberg *et al*. (2005) who found that 2-AG signals as an inverse agonist at this mutant, but are in agreement with Xiao *et al*. (2008). As observed with hCB_1 receptors, treatment with SR1 (200nM) did not potentiate EPSCs in hCB_{1a} expressing cells (Figure 5D).

hCB_{1b} receptor signalling is also more robust than hCB_1 receptor signalling

The hCB_{1b} splice variant, first described by Ryberg *et al*. (2005) is more conservative than hCB_{1a}, taking the form of a 33-amino-acid deletion of the middle of the amino terminus, leaving the first 21 amino acids intact (Figure 6A). Here, too, 2-AG was reported to act as an inverse agonist (Ryberg *et al*., 2005), though again not by Xiao *et al*. (2008). In our neuronal cultures, hCB_{1b} receptors signalled as effectively as rCB_1 receptors (Figure 6B,C). Also, 2-AG produced a substantial inhibition of the EPSC and treatment with the CB_1 receptor antagonist SR1 (200nM), did not potentiate EPSCs (Figure 6D).

Δ^9-THC and 11OHΔ^9-THC do not inhibit EPSCs via hCB_1 receptors or the splice variants

We have previously reported that the chief psychoactive component of marijuana and hashish, Δ^9-THC, activates mCB_1

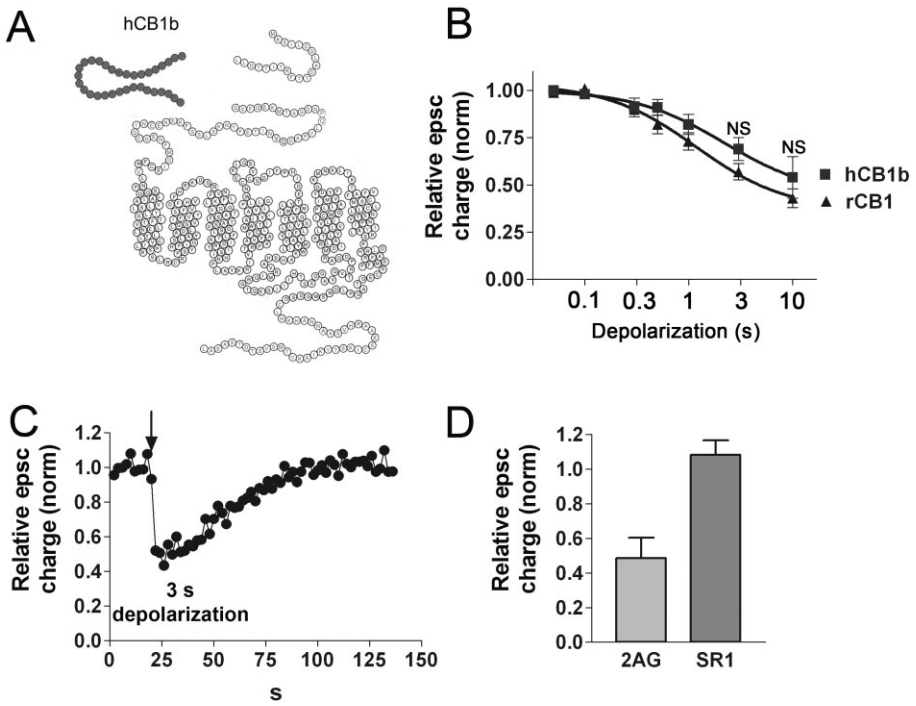

Figure 6

Signalling by hCB$_{1b}$ receptors is more robust than that by hCB$_1$ receptors. (A) Helixnet diagram schematic of hCB$_{1b}$ receptors highlights differences relative to hCB$_1$ receptors. The missing amino-terminus region is shown in darker symbols. (B) DSE depolarization–response curve, representing inhibition in response to different durations of depolarization (50 ms, 100 ms, 300 ms, 500 ms, 1 s, 3 s, 10 s) in neurones transfected with hCB$_{1b}$ receptors is not different from that in neurones transfected with rCB$_1$ receptors. NS, two-way ANOVA with Bonferroni *post hoc* test. (C) Typical DSE time course in response to 3 s depolarization. (D) Bar graph shows average responses to the endocannabinoid 2-AG (5 μM) and the CB$_1$ receptor antagonist SR1 (200 nM).

receptors in autaptic hippocampal neurones as a weakly efficacious, high-affinity ligand, activating the receptor sufficiently to induce internalization and desensitization with longer treatment but not sufficiently to acutely inhibit neurotransmission (Straiker and Mackie, 2005). It is possible that Δ^9-THC has a different activation profile at hCB$_1$ receptors and we therefore tested Δ^9-THC (1 μM) in neurones transfected with rCB$_1$ receptors, as well as with hCB$_1$ receptors and its splice variants (Figure 7A). Δ^9-THC did not inhibit EPSCs in cells expressing any of these receptors (Figure 7A)

Although Δ^9-THC is the chief psychoactive component found in marijuana and hashish (Gaoni and Mechoulam, 1964), it is rapidly metabolized into 11OHΔ^9-THC in the liver (Lemberger, 1972), making 11OHΔ^9-THC a relevant cannabinoid, particularly after oral ingestion when its levels rise above those of Δ^9-THC (Lemberger, 1972; Lemberger *et al.*, 1972). Interestingly, by some measures in mice, 11OHΔ^9-THC is more efficacious than Δ^9-THC (Christensen *et al.*, 1971). In our experiments, acute application of 11OHΔ^9-THC did not inhibit EPSCs in wild-type autaptic murine neurones (i.e. expressing mCB$_1$ receptors), or in neurones transfected with rCB$_1$, hCB$_1$, hCB$_{1a}$ or hCB$_{1b}$ receptors (Figure 7A). Like Δ^9-THC, 11OHΔ^9-THC blocked DSE in wild-type neurones (Figure 7B) and in rCB$_1$-transfected neurones (data not shown).

We have previously reported that while Δ^9-THC acts as an antagonist with respect to inhibiting synaptic transmission, it retains the ability to induce desensitization in autaptic neurones (Straiker and Mackie, 2005). We tested whether 11OHΔ^9-THC also desensitized the mCB$_1$ receptor in wild-type neurones and in CB$_1^{-/-}$ neurones transfected with rCB$_1$. Here we found that overnight treatment with 100 nM 11OHΔ^9-THC desensitized CB$_1$ signalling, resulting in a much-diminished DSE response profile (Figure 7C). Again, this is consistent with the action of a high-affinity, low-efficacy agonist.

Discussion

Our chief findings in this study are that in a neuronal environment capable of expressing DSE, transfection with hCB$_1$ receptors rescues signalling following genetic deletion of CB$_1$ receptors but does so much less robustly than transfection with rCB$_1$ receptors and that, in contrast, the receptor splice variants, hCB$_{1a}$ and hCB$_{1b}$, both fully rescue DSE. Our finding that 2-AG acts as an efficacious agonist at the splice variant receptors is consistent with the findings of Xiao *et al.* (2008), but not with those of Ryberg *et al.* (2005).

Although CB$_1$ receptors were initially cloned from rat (Matsuda *et al.*, 1990), much of the early work made use of hCB$_1$ receptors (cloned shortly thereafter; Gerard *et al.*, 1991) expressed in cell lines such as CHO and HEK293 cells. Using methods available at the time, these studies indicated that

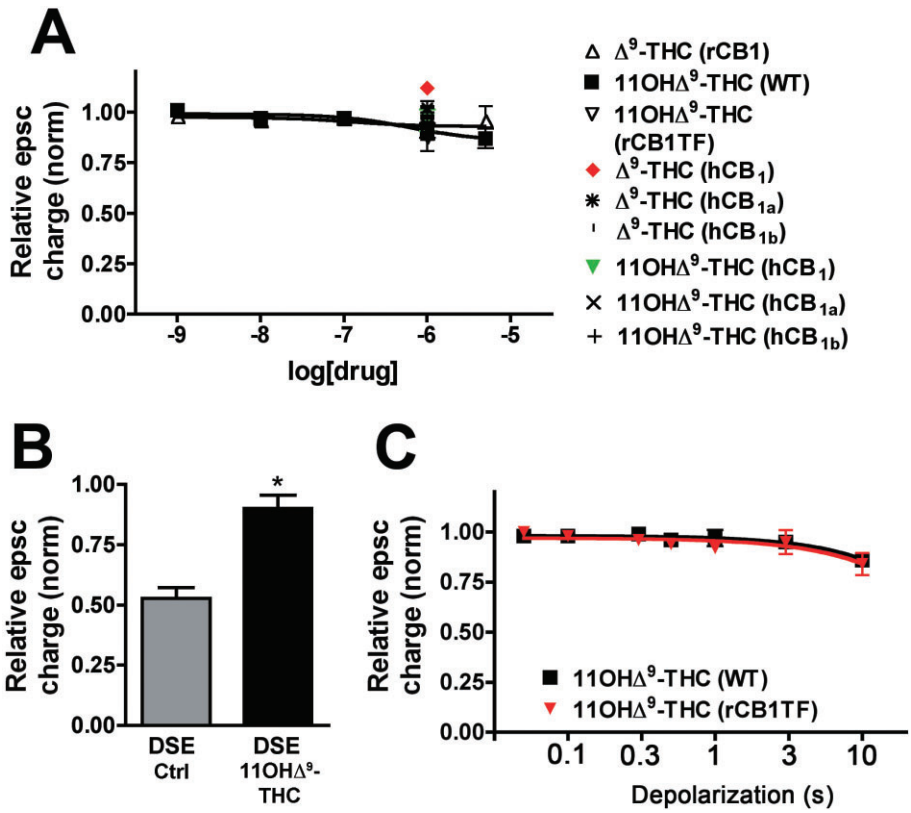

Figure 7

Δ^9-THC and 11OHΔ^9-THC do not inhibit EPSCs via hCB_1 receptors or the splice variants. (A) Dose–response curves for 11OHΔ^9-THC in wild-type (mCB_1) autaptic neurones, and Δ^9-THC in rCB_1-transfected $CB_1^{-/-}$ neurones, as well as 1 μM 11OHΔ^9-THC in rCB_1-transfected neurones, 1 μM Δ^9-THC in hCB_1-, hCB_{1a}- and hCB_{1b}-transfected neurones, in addition to 1 μM 11OHΔ^9-THC in hCB_1-, hCB_{1a}- and hCB_{1b}-transfected neurones. (B) Average DSE mediated by mCB_1 receptors before and after 11OHΔ^9-THC treatment. *$P < 0.05$ unpaired *t*-test. (C) DSE depolarization-response curves after overnight treatment with 11OHΔ^9-THC (100 nM) of WT neurones (mCB_1 receptors) or $CB_1^{-/-}$ neurones transfected with rCB_1 receptors.

hCB_1 receptors bound and were activated by a range of exogenous, synthetic and candidate endogenous cannabinoids (Felder *et al.*, 1993; 1995; Bouaboula *et al.*, 1995; Song and Bonner, 1996; Landsman *et al.*, 1997; Bonhaus *et al.*, 1998). With the cloning of the mouse CB_1 receptor (Chakrabarti *et al.*, 1995) and as CB_1-mediated synaptic plasticity, in the form of DSE/DSI and long-term depression (LTD) (Kreitzer and Regehr, 2001; Wilson *et al.*, 2001; Gerdeman *et al.*, 2002), was described in neuronal cultures and specific brain circuits, experimental inquiry shifted to more pliable rodent models (Kreitzer and Regehr, 2001; Ohno-Shosaku *et al.*, 2001; 2002a,b; Wilson and Nicoll, 2001). Although the hCB_1 receptor shares high (~97%) sequence similarity with mCB_1 and rCB_1 receptors (Matsuda *et al.*, 1990; Gerard *et al.*, 1991; Chakrabarti *et al.*, 1995), these few differences may nonetheless confer significant functional effects. Although we have not encountered any studies that compared rodent and human CB_1 receptor activation side by side, some studies were done under sufficiently similar conditions to merit comparison (Matsuda *et al.*, 1990; Gerard *et al.*, 1991; Felder *et al.*, 1992); these studies relied on CB_1 receptor-mediated inhibition of cAMP levels in response to forskolin activation and found similar EC_{50} and maximal inhibitions for the synthetic agonist CP55940 (Matsuda *et al.*, 1990; Gerard *et al.*, 1991; Felder *et al.*, 1992). However, the Gα subunit-dependent adenylyl cyclase inhibition may be qualitatively different from the Gβγ subunit-dependent modulation of neurotransmitter release machinery that is likely to underlie inhibition of neurotransmission in autaptic neurones (Sullivan, 1999; Vasquez and Lewis, 1999).

The ability to transfect a specific receptor into autaptic hippocampal neurones cultured from $CB_1^{-/-}$ mice allows direct comparison of the impact of subtle sequence differences in an endogenous neuronal setting. Our finding that hCB_1 receptors signal much less robustly than rCB_1 receptors under otherwise identical conditions is potentially significant, raising the possibility that synaptic transmission in humans is less sensitive to endogenous and exogenous cannabinoids. There is abundant evidence for ligand-specific actions at CB_1 and other GPCRs (Song and Bonner, 1996; Prather, 2004; Pineyro and Archer-Lahlou, 2007). But the question of species differences has not been systematically addressed for CB_1 receptors. While rodents serve as excellent model systems for the study of cannabinoid signalling, it is important to be aware of potential limitations, particularly since the functionality of human cannabinoid receptors is more socially

relevant. The decreased response in hCB$_1$ receptors was not limited to the endogenous agonist 2-AG, as the synthetic cannabinoid WIN also signalled poorly even at 1 μM, a concentration that robustly inhibits synaptic transmission in autaptic hippocampal neurones (Straiker and Mackie, 2005). AEA also signalled poorly, despite a report of a greater AEA potency at hCB$_1$ receptors (Ryberg *et al.*, 2005). Δ^9-THC failed to activate hCB$_1$ or rodent CB$_1$ receptors to inhibit EPSCs. We were also able to examine potential differences in signalling between Δ^9-THC and its chief active metabolite, 11OHΔ^9-THC, with the finding that in rCB$_1$-transfected neurones both phytocannabinoids acutely antagonized endocannabinoid signalling but will desensitize CB$_1$ receptor signalling following prolonged treatment.

Cannabinoid CB$_1$ receptors have been shown to mediate a remarkable variety of signalling pathways and effectors (Kano *et al.*, 2009). Even in autaptic neurones, differential activation can result in short-term modulation lasting tens of seconds or long-term inhibition lasting tens of minutes or longer (Straiker and Mackie, 2005; Kellogg *et al.*, 2009). The possibility of alternative mRNA splicing allows greater flexibility and adaptability for the human cannabinoid receptor signalling system. Although the receptor splice variants hCB$_{1a}$ and hCB$_{1b}$, were functional in our neuronal cultures, our results differed substantially from those of Ryberg *et al.*, (2005), using GTPγS assays to examine activation of hCB$_{1a/b}$ receptors. Rather than 2-AG acting as an inverse agonist (Ryberg *et al.*, 2005), we found 2-AG to be an efficacious agonist for both hCB$_{1a}$ and hCB1$_b$ receptors. Furthermore, both splice variants rescued DSE, signalling much more robustly than hCB$_1$ receptors and almost as effectively as rCB$_1$receptors. In the case of hCB$_{1b}$ receptors, the difference in signalling may be due to relatively higher receptor expression levels. The significance of this is difficult to assess in part because to date no analogous rodent splice variants have been reported, thereby placing substantial limitations on understanding the behavioural consequences of the splice variants. In principle, however, the differences in maximal signalling as assessed by DSE inhibition raise the possibility of a transcriptional switch from a low-efficacy to a high-efficacy cannabinoid receptor, as needed. Ryberg *et al.* (2005) reported that both splice variants were detected in an assortment of tissues, but at low levels. However, they point out that these levels are comparable with levels of hCB$_1$ receptors in tissues such as spleen, and if degradation of hCB$_1$ splice variants is slow, the protein levels of these splice variants may be high. In addition, particular splice variants may be preferentially expressed in specific cellular subpopulations, increasing the complexity of endocannabinoid signalling. This will be an important possibility to pursue. As a final caveat, it should be noted that although the ability to observe proteins from different species in a uniquely controllable environment is a powerful tool, it suffers from the limitation that the cellular milieu may itself be a determinant of the response. Thus, hCB$_1$ receptors may signal very differently in murine neurones than they do in human neurones. If so, this would be of considerable interest and will be an interesting avenue of future investigation.

In summary, we found that hCB$_1$ receptors and the splice variants hCB$_{1a}$ and hCB$_{1b}$ all functionally rescued DSE in autaptic hippocampal neurones from CB$_1^{-/-}$ mice. However, the signalling of hCB$_1$receptors, but not its splice variants hCB$_{1a}$ or hCB$_{1b}$, was significantly diminished relative to that of rCB$_1$receptors, a finding that may have implications for the use of rodent models for studies of CB$_1$ receptor function related to human disease and therapy. We have also found that in this neuronal environment, the endocannabinoid 2-AG engages the splice variant receptors as an agonist. Taken together, our results invite a closer examination of species-specific relative functionality of CB$_1$ receptors and any splice variants that may be encountered.

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Conflict of interest

The authors have no competing financial interests in relation to the work described.

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